Evaluation of repetitive extragenic palindromic-polymerase chain reaction and denatured gradient gel electrophoresis in identifying *Salmonella* serotypes isolated from processed turkeys

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ABSTRACT The current study was conducted to determine the usefulness of 2 molecular techniques, automated repetitive extragenic palindromic-PCR (REP-PCR) and denaturing gradient gel electrophoresis (DGGE), to identify *Salmonella* serotypes of poultry origin. *Salmonella* continues to be a foodborne pathogen of principal concern in the United States. The interspersed conserved repetitive sequence of the bacterial genome and the 16-23S rDNA intergenic spacer region were amplified for REP-PCR and DGGE, respectively. Fifty-four *Salmonella* isolates from 2 turkey processing plants (A and B) were used for this comparison. Serotypes consisted of Brandenburg, Derby, Hadar, and Typhimurium, with n = 6, 21, 12, and 15, respectively. The REP-PCR was fully automated, whereas DGGE was run on an acrylamide gel and the image was captured digitally. Both dendrograms were created using the unweighted pair group method with arithmetic average. There were more variations in percentage similarity in DGGE when compared with REP-PCR. The banding patterns were more distinct and uniform in the REP-PCR group than with DGGE. The results from the REP-PCR were generated within 1 h, whereas the DGGE required approximately 1 d to run. These data suggest that DGGE and REP-PCR are useful tools for identifying *Salmonella* serotypes isolated from poultry production or processing environments. In addition, REP-PCR is more rapid, may have a higher discriminatory power, but may be less cost-effective than DGGE. However, more research may be needed to validate this argument. Both DGGE and REP-PCR displayed high sensitivity in discriminating among *Salmonella* serotypes and either method could be considered as an alternative to more expensive and time-consuming conventional antibody-based serotyping methodologies.

Key words: *Salmonella*, repetitive extragenic palindromic-polymerase chain reaction, denaturing gradient gel electrophoresis, serotype, poultry

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INTRODUCTION

Foodborne *Salmonella* infections represent a very significant threat to human health both within the United States and worldwide (Chang, 2000; Jørgensen et al., 2002; Liljebjelke et al., 2005). Globally, it is estimated that there are over 21.6 million cases of typhoid *Salmonella* documented annually (Crump et al., 2004). Nontyphoidal *Salmonella* infections are clearly more common and are linked to over 1.3 billion cases and approximately 3 million deaths annually (Pang et al., 1995). In the United States, between 1996 and 1999, foodborne illnesses from *Salmonella* Typhi were estimated to be fewer than 700 cases. Of these, 492 persons were hospitalized and 3 deaths occurred. Nontyphoidal *Salmonella* infection in the United States is estimated to result in 1.34 million cases, of which 16,430 persons were hospitalized and 553 deaths were reported (Mead et al., 1999). Annually, salmonellosis costs US $2.4 billion, resulting from medical costs, loss of productivity, and premature deaths (USDA-ERS, 2005).

Salmonella has been frequently reported in the products of plants and animals, whereas poultry meat and eggs are considered to be a major vehicle for the transmission of *Salmonella* to humans (Li and Mustapha, 2002; Capita et al., 2003; Vadhanasin et al., 2004). Several routes for contamination among commercial poultry have been established, including breeder flocks

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(Liljebjelke et al., 2005), hatcheries (Byrd et al., 1999), grow-out farms (Caldwell et al., 1994; Bailey et al., 2001), feed (Maciorowski et al., 2005), transport or live-haul (Slader et al., 2002), and processing (Corry et al., 2002).

Consumers are becoming more health conscious with regard to food choices (Guo et al., 1999). The media could be credited for keeping consumers informed about foodborne pathogen outbreaks and the recalls of meats and products. Recalls are very costly to the poultry industry. As a quality control measure, several intervention strategies have been employed by commercial integrators, especially at processing facilities, with the aim of reducing pathogens on the final product. Hence, the food industry is continuously exploring new pathogen detection methods that need to be inexpensive, fast, and reliable, to augment food safety initiatives (Guo et al., 1999).

Conventional methods such as serotyping, phage typing, antibiotic resistance (R-type), biotyping, antibiogram, and bacteriocin identification (Sader et al., 1995; Cooke et al., 2007) are known methods for testing foodborne pathogens. These methods are laborious, nonsensitive, slow, and often unreliable. Of the conventional methods, serotyping and phage typing are the most widely used to type *Salmonella.* Serotyping of bacteria is based on antigen-antibody interaction. *Salmonella* has 2 surface antigens, somatic (O) and flagella (H), which are used to divide the bacteria into serogroups (Grimont et al., 2000; Andrews and Baumler, 2005). Phage typing is based on the principle of lysing bacteria with bacteriophages. *Salmonella* serotypes such as *Salmonella* Typhi and *Salmonella* Typhimurium possess a surface envelope (Vi) antigen that can be identified using specific phages. Phage typing is done by using an internationally recognized set of phages to identify the bacteria. According to Wang et al. (2008), *Salmonella* Typhimurium with a set of 34 phages allows the identification of 207 phage types.

Deoxyribonucleic acid fingerprinting techniques for distinguishing bacterial isolates have been shown to be fast, sensitive, specific, highly reproducible, and less labor-intensive than conventional methodologies (Whyte et al., 2002; Oliveira et al., 2003). Aside from the demonstrated positives, each method has its limitations (Agarwal et al., 2002). Molecular techniques have been widely accepted as the alternative to conventional methods in many research settings (Jitrapakdee et al., 1995; Johnson and Clabots, 2000). Polymerase chain reaction-based techniques such as denaturing gradient gel electrophoresis (**DGGE**; Ercolini, 2004), repetitive extragenic palindromic-PCR (**REP-PCR**; Jonas et al., 2003), real time-PCR (Hein et al., 2006), and pulsed field gel electrophoresis (Whyte et al., 2002; Oliveira et al., 2003) are some of the current methods used to discriminate among bacterial species, serotypes, and strains. The present study will focus exclusively on the use of DGGE and REP-PCR.

Muyzer et al. (1993) were the first to apply DGGE to microbial ecology studies. The primers target the conserved region that lies next to the hypervariable V3 region of the 16S rDNA (Muyzer et al., 1993; Hume et al., 2003). Use of the internal spacer region between the 16S rDNA and the 23S rDNA has been well exploited in prokaryotic organisms such as *Salmonella* (Chiu et al., 2005). Denaturing gradient gel electrophoresis separates DNA fragments that are identical in length but have different nucleotide sequences (Muyzer et al., 1993). Double-stranded DNA migrates along the increasing denaturing gradient and melts in a discrete so-called melting domain (Muyzer et al., 1998). As the domain approaches the lowest temperature, the double helix partially melts and migration ceases. As a result, DNA molecules with different nucleotide sequences will migrate different distances along the gel (Muyzer et al., 1993, 1998; Ercolini, 2004). The addition of a 40–50 GC-rich (GC clamp) sequence to the 5′ end of one of the primers increases the temperature requirement of that fragment (Roelfsema and Peters, 2005).

Repetitive extragenic palindromic-PCR targets the highly conserved, interspersed, repetitive elements found at several sites within the eukaryotic and prokaryotic genome (Healy et al., 2005; Frye and Healy, 2006). The conserved region that lies close to the repeated elements differs according to size, thus producing fragments of varying length, evident via agarose gel electrophoresis (Foley and Grant, 2007). The fragment size provides a distinct fingerprinting profile for the organism (Frye and Healy, 2006), which forms the basis for band comparison (Foley and Grant, 2007). Repetitive extragenic palindromic-PCR has the discriminatory power to identify bacteria (*Bacillus subtilis*, *Bartonella*, *Escherichia coli*, and *Salmonella*) at the subspecies and strain level (Beyer et al., 1998; Olive and Bean, 1999; Healy et al., 2005).

Recently, manual REP-PCR has been replaced by an automated system. The DiversiLab system (Bacterial Barcodes Inc., Athens, GA) separates PCR amplicons on polyacrylamide microfluidics chips and a Web-based program is used to create customized output (Healy et al., 2005; Frye and Healy, 2006). The system has been reported to be very time-efficient and highly reproducible among laboratory technicians, microfluidics chips, DNA concentrations, laboratory equipment, and different culture conditions (Healy et al., 2005).

To the best of our knowledge, only a limited number of studies have compared DGGE and REP-PCR in characterizing foodborne pathogens. Therefore, there is a need to explore the discriminatory powers of REP-PCR and DGGE in foodborne pathogen characterization. The current study was conducted to determine the usefulness of 2 molecular techniques, REP-PCR and DGGE, to identify *Salmonella* serotypes of poultry origin. Additionally, the automated REP-PCR and DGGE were compared as diagnostic tools for their abilities to discriminate *Salmonella* serotypes.

MATERIALS AND METHODS

Fifty-four *Salmonella* isolates collected from 2 turkey processing facilities (A and B) were used for this study. The serotypes present among these isolates were Brandenburg, Derby, Hadar, and Typhimurium [n = 6 (all plant A), 21 (14 plant A, 7 plant B), 12 (5 plant A, 7 Plant B), and 15 (all plant B), respectively]. All isolates were previously typed at the USDA-Animal and Plant Health Inspection Service, National Veterinary Services Laboratory (**NVSL**), in Ames, Iowa. After their initial isolation, all isolates were stored at −80 C in tryptic soy broth containing 20% (vol/vol) glycerol until analysis in this investigation.

DNA Extraction

Approximately 10 μL from the frozen stock culture was streaked onto brilliant green agar (**BGA**) supplemented with 25 μg/mL of novobiocin. The BGA plates were incubated for 18 to 24 h at 37°C. Bacterial colonies from each plate were used for DGGE and REP-PCR DNA extraction.

DGGE. A colony for typing by DGGE was chosen from each BGA plate and placed in 200 μL of sterile Tris-EDTA buffer (10 m*M* Tris-1 m*M* EDTA, pH 8.0) and the bacterial cells were lysed by heating in a boiling water bath for 15 min. The isolates were chilled and centrifuged at 10,000 × *g* for 10 min to separate out cellular particles, and the supernatant was removed. Genomic DNA concentrations were measured spectrophotometrically (Nanodrop ND-1000, NanoDrop Technologies, Wilmington, DE), standardized to 15 ng/μL, and stored at −20°C until needed for DNA amplification.

REP-PCR. Approximately 2 μL (inoculation loop) of bacterial cells was used for DNA extraction for REP-PCR. The extraction was performed by exposing the cells to microbead beating, following the protocol of the Mo Bio Ultraclean Microbial DNA Isolation Kit (Mo Bio Laboratory Inc., Carlsbad, CA) and according to the manufacturer's recommendations. After extraction, DNA was measured spectrophotometrically (Nanodrop ND-1000), adjusted to 25 ng/μL, and stored at −20°C before amplification.

PCR Amplification

DGGE. The primers used were previously reported by (Bakshi et al., 2002) with slight modification. The amplification of the target region was achieved using 2 primers (50 pmol of each): forward G_1 5′-GAAGTCGTAACAAGG-3′ and reverse L_1 5′-CAAGGCATCCACCGT-3′ (Integrated DNA Technologies, Coralville, IA). A GC-rich 40-base clamp (Sheffield et al., 1989; Muyzer et al., 1993) 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3′ was attached to the 5′ end of the G_1 primer. The primers were combined with a commercial Jump Start Red-Taq Ready Mix (Sigma Chemical Company, St. Louis, MO), 1 μL of BSA (10 mg/mL), 2 μL of DNA template (15 ng/μL), and deionized water to make a final 50-μL reaction volume. Polymerase chain reaction of DNA was performed in a PTC-200 thermal cycler (MJ Research Inc., Watertown, MA). The PCR protocol was adapted from a previous study (Hume et al., 2003): 1) initial denaturing at 94.9°C for 2 min; 2) subsequent denaturation at 94°C for 1 min; 3) annealing at 67°C for 45 s, −0.5°C per cycle (touchdown to minimize spurious by-products; Don et al., 1991; Wawer and Muyzer, 1995); 4) extension at 72°C for 2 min; 5) repeat steps 2 to 4 for 17 cycles; 6) denaturation at 94°C for 1 min; 7) annealing at 58°C for 45 s; 8) repeat steps 6 to 7 for 12 cycles; 9) extension at 72°C for 7 min; and 10) held at 4°C for the final stages.

REP-PCR. Amplification was done using the DiversiLab fingerprint kit (Wise et al., 2009) and following the manufacturer's recommendations. Briefly, a master mix containing 18 μL of REP-PCR MM1, 2.5 μL of GeneAmp 10× PCR buffer (Applied Biosystems, Foster City, CA), 2 μL of Primer Mix LL, 0.5 μL of AmpliTaq DNA polymerase, and 2 μL (50 ng) of DNA were added to a final volume of 25 μL. Amplification of DNA was performed in a PTC-200 thermocycler. The PCR conditions were initial denaturation at 94°C for 2 min; subsequently, a 35-cycle program of denaturing at 94°C for 30 s; annealing at 45°C for 30 s; and extension at 70°C for 90 s. Last, a single cycle for 3 min at 70°C was added to the final cycle.

Gel Electrophoresis

DGGE. The procedure was performed according to the method previously reported (Muyzer et al., 1993) with some modification (Hume et al., 2003). The PCR products were separated on an 8% (vol/vol) polyacrylamide:bisacrylamide gel (37.5:1) with a denaturing gradient of 35 to 45% (100% denaturing acrylamide; 7 *M* urea and 40% deionized formamide). Four microliters of PCR amplicons was mixed with an equal volume of 2× loading buffer [0.05% (wt/vol) bromophenol blue, 0.05% (wt/vol) xylene cyanol, and 70% (vol/vol) glycerol] and 7 μL was placed in each sample well (20-well comb). Gel electrophoresis was performed in a DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA) using 1× Tris-sodium acetate-EDTA buffer (20 m*M* Tris, 10 m*M* sodium acetate, and 0.5 m*M* EDTA, pH 7.4) and ran at 59°C for 17 h at 60 V (Hume et al., 2003). After electrophoresis, the gels were stained using SYBR Green (1:10,000 dilution) in 1× Tris-sodium acetate-EDTA buffer for 30 min and destained using distilled water.

REP-PCR. The DNA fragments were separated on a 1.5% acrylamide gel microfluidics chip following the instructions of the manufacturer (Mo Bio Laboratory Inc.). Briefly, 5 μL of DNA marker was added to each

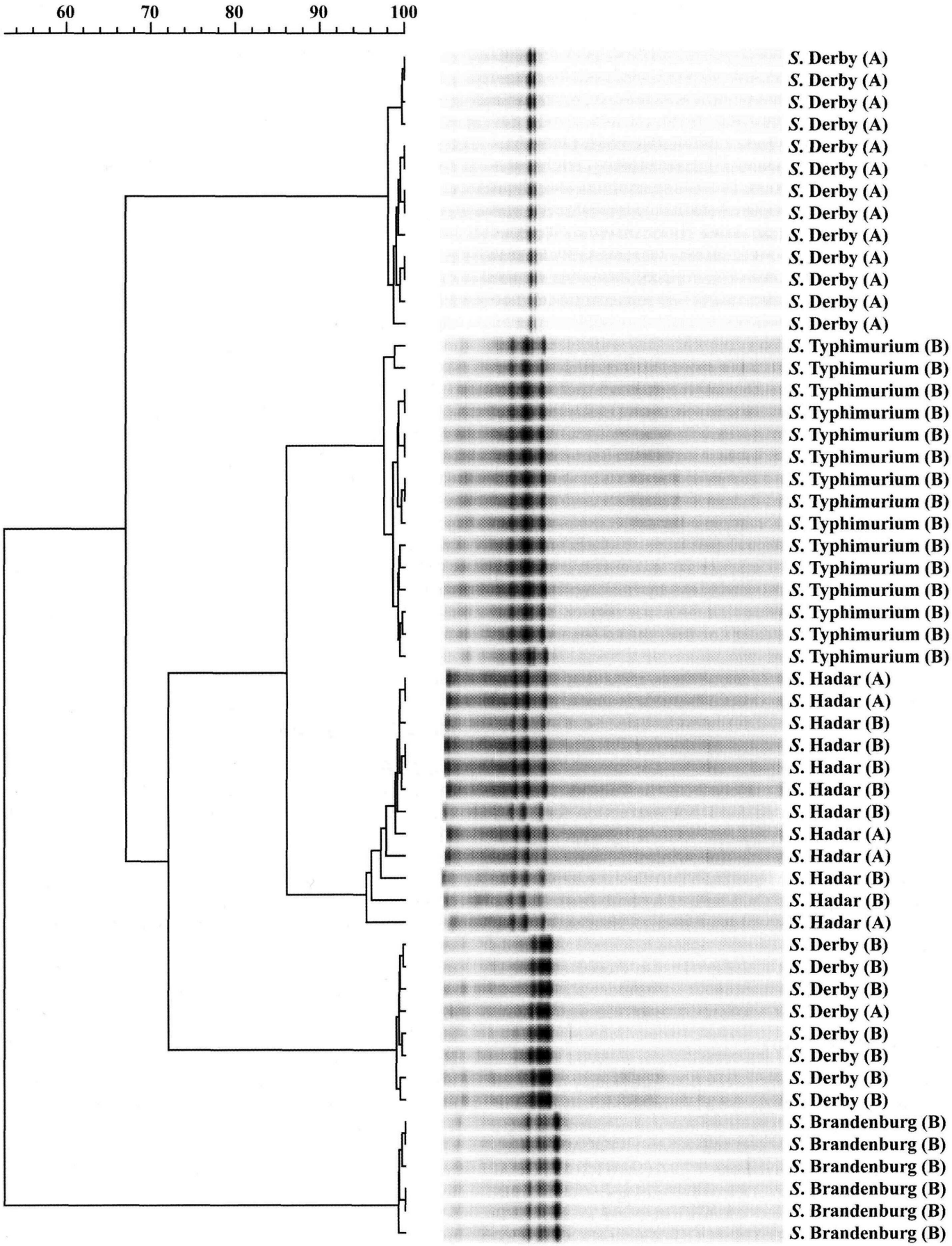

Figure 1. Dendrogram showing denaturing gradient gel electrophoresis profiles of 54 *Salmonella* isolates recovered from 2 turkey processing plants (A and B). Percentage similarity coefficient is indicated by the bar above the dendrogram; ≥92% are very related or the same, 85 to 91% are similar, 80 to 84% are somewhat similar, and ≤79% are unrelated (Dunkley et al., 2007).

of 12 wells and also to the ladder well on the microfluidics chip (LabChip Device, Caliper Technologies Inc., Hopkinton, MA). Next, 1 μL of PCR product was added to the same wells. Finally, the microfluidics chip was vortexed for 1 min then placed for approximately 1 h in a model B 2100 bioanalyzer (Agilent Technologies Inc., Palo Alto, CA) for PCR fragment separation (Healy et al., 2005).

DNA Fingerprint Analysis

DGGE. The DGGE gel images were digitalized (Alpha Imager, Alpha Innotech Corporation, San Leandro, CA). Additionally, DNA fingerprint patterns were analyzed and a dendrogram was generated using Molecular Analysis Fingerprinting Software, Version 1.6 (Bio-Rad Laboratories) and using the Dice similarity coefficient (**SC**) and the unweighted pair group method using arithmetic averages for clustering.

REP-PCR. Deoxyribonucleic acid analysis was performed using the DiversiLab software (version 2.1.66). The software created customized reports, including a dendrogram, electropherograms, virtual gel images, and scatter plots (Healy et al., 2005). A DNA fingerprint profile of each lane was compared pairwise against all of the other lanes. Pearson correlation coefficient was used to determine the percentage of similarity among the different band patterns and the unweighted pair group method using arithmetic averages was used to create the dendrogram of the different clusters (Johnson and Clabots, 2000; Healy et al., 2005).

RESULTS

DGGE

Figure 1 represents the dendrogram of 54 *Salmonella* isolates from 2 commercial turkey processing facilities located in 2 distinct geographical locations within the United States. The processing plants from which the isolates were recovered are labeled as plant A and plant B. The 54 *Salmonella* isolates consisted of 4 distinct serotypes: Brandenburg, Derby, Hadar, and Typhimurium. At 90% SC, there were 5 genotypic clusters observed among the isolates (Table 1). *Salmonella* Hadar contained 12 isolates and displayed genetic similarity of 95.4%. All the *Salmonella* Typhimurium isolates were collected within plant B and showed a consistent band pattern with 97.6% SC. The 6 *Salmonella* Brandenburg DNA profiles were genetically identical at 99.2% SC. The 2 band patterns of the *Salmonella* Derby isolates were very diverse and displayed unrelated profiles. All of the *Salmonella* Derby isolates from plant B along with 1 isolate from plant A exhibited a 99.0% SC. Similarly, the remaining 13 *Salmonella* Derby isolates from plant A were 98.0% related. Overall, the relatedness of *Salmonella.* Derby between the 2 plants was heterogeneous and exhibited only 67.7% SC.

Table 1. Percentage similarity coefficients of *Salmonella* serotypes recovered from 2 turkey processing plants (A and B) and analyzed using denaturing gradient gel electrophoresis (DGGE) and repetitive extragenic palindromic-PCR (REP-PCR)

	Percentage similarity coefficient	
Salmonella serotype	DGGE	REP-PCR
Brandenburg (plant B)	99.2	96.7
Derby (plant A)	98.0	92.5
Derby (plant B)	99.0	94.0
Derby (plant A and B)	67.7	76.7
Hadar (plant A)	95.4	90.1
Typhimurium (plant B)	97.6	88.2

REP-PCR

The DNA fingerprinting profiles for the 54 isolates subjected to REP-PCR are shown in Figure 2. At 90% SC, there were 5 major clusters among the *Salmonella* isolates (Table 1). Collectively, *Salmonella* Brandenburg isolates were approximately identical as they grouped at 96.7% SC, which is slightly lower than groupings observed with DGGE (99.2% SC). The greatest variation was observed in *Salmonella* Typhimurium and these isolates were subdivided into 6 groups. Overall, the *Salmonella* Typhimurium isolates had the poorest correlation (88.2% SC) among all of the serotypes. Two main groups were detected with *Salmonella* Hadar isolates: the first 4 isolates had 94.8% SC and the second group had 96.8% SC; however, both groups were different at SC 90.1%. Interestingly, all of the *Salmonella* Derby isolates from plant A with the exception of one were segregated from isolates from plant B. Only slight variations were noted in both groups and plant A isolates were 92.5% similar. All of the plant B *Salmonella* Derby, in addition to 1 plant A isolate, were related at 94.0% SC. Both groups of isolates produced distinguishable band patterns and were only genetically related at 76.7% SC.

DISCUSSION

The current study evaluated the discriminatory powers of 2 well-characterized molecular-based genotyping techniques, REP-PCR and DGGE. A search of PubMed revealed only limited data contrasting REP-PCR and DGGE with organisms such as bifidobacteria (Masco et al., 2005) and lactobacillus, but limited work was discovered focusing on *Salmonella* or other enteric pathogens. One possible reason for the lack of comparisons between DGGE and REP-PCR could stem from that fact that many laboratories have already invested in equipment and other techniques (Weigel et al., 2004) and as such may lack the funds to acquire new equipment required for other PCR-based methods of genotyping. When it comes to *Salmonella* typing, the gold standard is the reliable antibody serotyping method

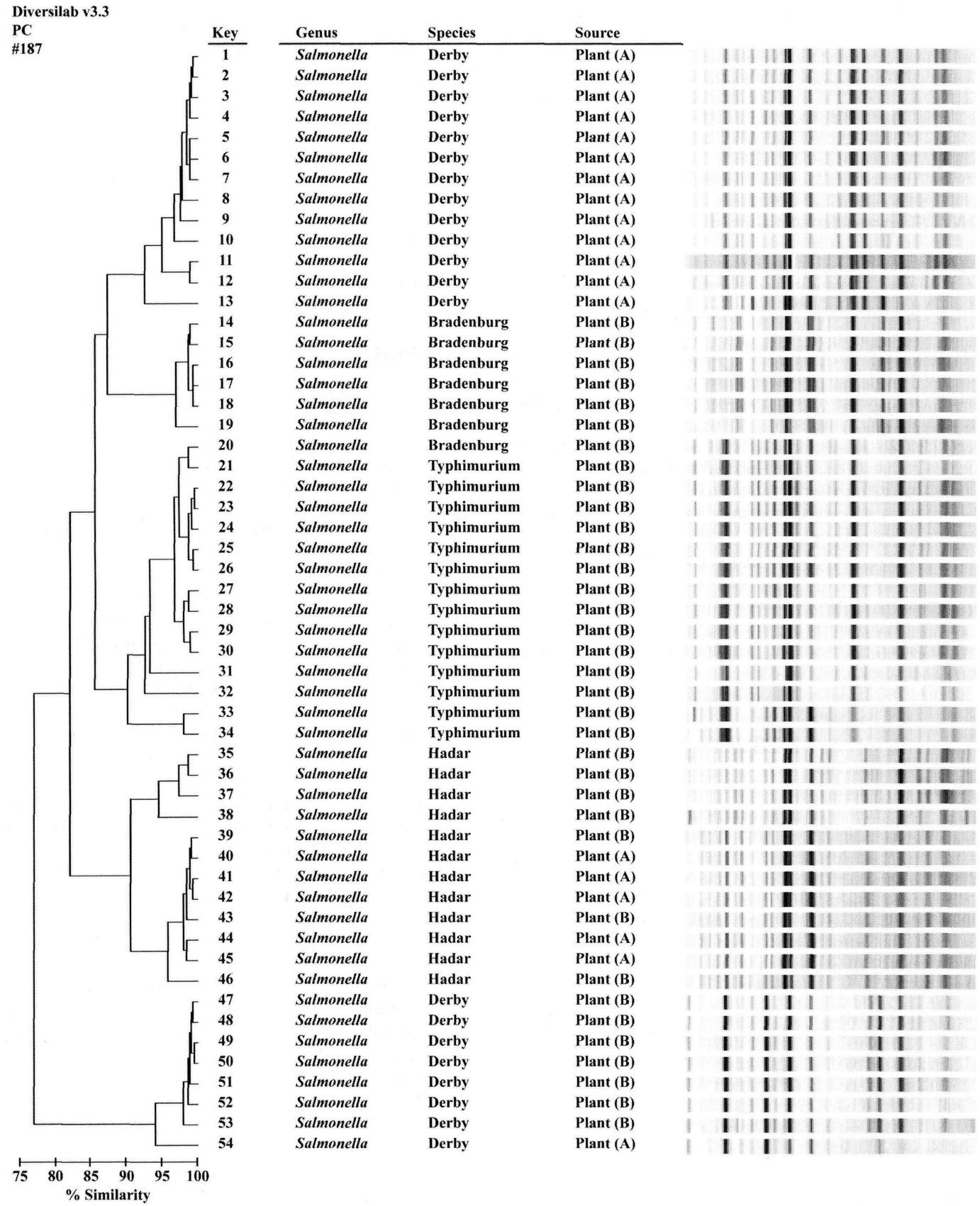

Figure 2. Dendrogram showing the repetitive extragenic palindromic-PCR DNA profiles of 54 *Salmonella* isolates recovered from 2 turkey processing plants (A and B). Percentage similarity coefficient is indicated by the bar below the dendrogram; ≥92% are very related or the same, 85 to 91% are similar, 80 to 84% are somewhat similar, and ≤79% are unrelated (Dunkley et al., 2007).

and with no consensus methodology developed for molecular typing of *Salmonella* serovars.

In our investigation, REP-PCR typically generated more band fragments compared with DGGE, thus creating potentially more variability among the fingerprinting profiles. The greater number of bands displayed by REP-PCR compared with DGGE may explain the high degree of variability we observed. Another investigation revealed that REP-PCR is very sensitive and highly discriminatory among bacterial strains (Beyer et al., 1998). Similarly, DGGE displayed sensitivity in foodborne pathogens recovered from several food products (Ercolini, 2004). Both genotypic methods were able to discriminate between the *Salmonella* Derby isolates recovered from the 2 processing plants sampled in this study, located in 2 distinct geographical locations in the United States. In addition, the clustering of the *Salmonella* Derby from plant A (outlier) with those plant B isolates was differentiated by both fingerprinting techniques. One of the problems often experienced with DGGE is that each gel has to be properly aligned to effectively analyze several gels in a comparison. In addition, REP-PCR has limitations; air bubbles in the microfluidics chip can cause dark smears to develop, which may cover DNA bands, forcing the sample to be reevaluated.

Both molecular methods rely heavily on a large database of *Salmonella* DNA fingerprints for usefulness in identifying unknown isolates. To generate the database, the isolates first have to be serotyped, which could be costly because each *Salmonella* isolate costs US $35 when typed at USDA-Animal and Plant Health Inspection Service, NVSL, in Ames, Iowa. Another alternative to reduce cost and generate an extensive, representative database is to collaborate with other researchers and acquire isolates that have previously been serotyped.

In our hands, PCR-based DGGE was more economical than REP-PCR in identifying large numbers of *Salmonella* isolates. Without adding the cost of labor, our laboratory calculated the cost per sample using DGGE to be US $12, whereas it cost $27 for a similar evaluation using REP-PCR. One of the most significant advantages to REP-PCR was the reduced time required to analyze a sample. During this study, REP-PCR analyses required 1 h to complete 13 isolates, whereas the DGGE commonly required 17 h to run 2 gels per setup with 20 isolates per gel. In contrast to molecular typing, serotyping can take up to weeks to receive confirmation on an isolate. The reason for this is because serotyping is done primarily in a large reference laboratory such as the NVSL and requires numerous reagents to properly type an isolate. As such, it is often not economical for small laboratories to invest in *Salmonella* serotyping.

Collectively, both techniques were highly discriminatory among *Salmonella* isolates. However, REP-PCR showed a higher variability in the amplicon patterns compared with DGGE, suggesting that REP-PCR was able to detect slight variations in the DNA fragments. Although PCR-based techniques are known to show higher sensitivity than antibody-based methods, not much work has been done to evaluate sensitivity with reference to *Salmonella* typing. Both DGGE and REP-PCR displayed high sensitivity in discriminating among *Salmonella* serotypes and either method could be considered as an alternative to more expensive and time-consuming conventional antibody-based serotyping systems.

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